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Triazole fungicides and the selection of resistance to medical triazoles in the opportunistic mould *Aspergillus fumigatus*

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Abstract

Azole resistance is an emerging problem in the opportunistic mould *Aspergillus fumigatus*. The triazoles are the most important agents for the management of *Aspergillus* diseases in humans. Selection for acquired resistance may occur in the hospital setting through exposure to high doses of azoles during azole therapy, but evidence is accumulating that *A. fumigatus* may become resistant to medical triazoles through environmental exposure to fungicides. The recovery of *A. fumigatus* isolates resistant to the medical triazoles from azole-naive patients as well as from the environment strongly indicates an environmental route of resistance selection. Molecule alignment studies have identified five fungicides that share a very similar molecule structure with the medical triazoles, and thus may have selected for mechanisms that confer resistance to both groups of compounds. It is important to explore further the presumed fungicide-driven route of resistance selection in order to implement effective preventive measures as the prevalence of azole resistance in *A. fumigatus* continues to increase and causes major challenges in the management of azole-resistant *Aspergillus* diseases.

Keywords: *Aspergillus fumigatus*; triazoles; resistance; demethylase inhibitors; aspergillosis

1 INTRODUCTION

The mould *Aspergillus fumigatus* is a saprophytic fungus that can reproduce through an asexual and sexual cycle. The fungus is not phytopathogenic, but may cause a spectrum of diseases in humans, depending on the state of the underlying immune function. In patients with normal immune function, conditions such as aspergilloma may occur.[1] Aspergilloma is characterised by growth of *Aspergillus* in a pre-existing cavity, commonly in the lung, usually owing to a previous tuberculosis. In patients with a hyperactive immune system, allergic conditions may occur, such as allergic bronchopulmonary aspergillosis (ABPA). Finally, immunocompromised patients may be at risk of developing invasive aspergillosis, including patients with hematologic malignancy, solid organ transplant recipients and critically ill patients.[1] In patients with invasive aspergillosis, the airborne conidia that are inhaled cannot be efficiently removed by alveolar macrophages. The conidia germinate and exhibit invasive growth into the surrounding tissues. This may cause necrosis and bleeding owing to concomitant coagulopathy, and, if left untreated, the fungus will disseminate from the lung to other tissues. The morbidity and mortality associated with invasive aspergillosis is significant and ranges between 20 and 65%, depending on the underlying immune status and the presence of disseminated disease.[2] The incidence rates vary from below 1% in autologous hematopoietic stem cell transplant (HSCT) recipients to up to 27% in allogeneic HSCT patients.[3] The incidence in critically ill patients was found to be 2.7–6.3%.[3] *Aspergillus fumigatus* is the most frequently encountered species, followed by *A. flavus* and *A. niger*. It is generally accepted that most immunocompromised patients become infected outside the hospital, and that disease becomes clinically evident during hospitalisation, when intensive immunosuppressive treatment is administered.[4–6]

Only two classes of antifungal compounds are clinically licensed for primary therapy of invasive aspergillosis, the polyenes and the azoles. The class of azoles is the main group of compounds that are used for the management of aspergillus diseases, and comprises three compounds with activity against *Aspergillus* species, i.e. itraconazole, voriconazole and posaconazole. Voriconazole is recommended for the primary therapy of invasive aspergillosis,[7] and posaconazole was shown to reduce the number of invasive fungal infections in neutropenic patients with acute myeloid leukemia, with myelodysplastic syndrome and in patients with severe graft-versus-host disease when administered prophylactically.[8,9]

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Itraconazole is commonly used for the management of non-invasive aspergillus diseases. In patients who fail to respond to first-line therapy, it is common practice to change drug class, i.e. from azole to polyene or vice versa. The echinocandin caspofungin is also clinically licensed for salvage therapy of invasive aspergillosis.

2 ACQUIRED AZOLE RESISTANCE

2.1 Epidemiology

In clinical microbiology, *in vitro* susceptibility testing of *Aspergillus* species has not routinely been performed, as these are considered to be uniformly susceptible to the medical triazoles. However, there are an increasing number of reports of acquired resistance in *A. fumigatus*. The authors recently reported the rapid emergence of azole resistance in *A. fumigatus* isolates cultured from patients with invasive aspergillosis.[10,11] In the Netherlands, the first clinical azole-resistant *A. fumigatus* isolate was cultured in 1998,[12] and since then the prevalence has increased. A recent national survey showed that azole resistance was widespread in the Netherlands, with an overall prevalence of 5.3%.[13] In this surveillance study, all *Aspergillus* isolates that were cultured from clinical specimens were subcultured on Sabouraud agar supplemented with itraconazole. Any isolate that grew in the presence of itraconazole was further analysed by *in vitro* susceptibility testing and determination of the underlying resistance mechansim.[13] Azole resistance was found in clinical isolates that were recovered from all participating hospitals.[13] *In vitro* susceptibility testing showed that all isolates were resistant to itraconazole (Fig. 1). However, the activity of voriconazole and posaconazole was also reduced compared with wild-type control isolates. According to the recently proposed breakpoints,[14] 79% of these isolates were resistant to voriconazole, and 17% to posaconazole.[13]

2.2 Clinical implications of resistance

The clinical implications of azole resistance are significant, as it poses challenges for the management of patients with *Aspergillus* diseases. Preclinical evidence suggests that the decreased azole susceptibility, or increase in minimum inhibitory concentration (MIC), is an important factor for drug efficacy. Animal models were used to investigate whether the efficacy of voriconazole and posaconazole was reduced in mice infected with isolates with increased MICs of these drugs.[15,16] These studies showed that there was a clear decrease in survival in animals infected with isolates with an increased MIC, indicating that the MIC is an important factor in the probability of azole treatment success.[15,16] Clinical experience supports the results of the animal experiments. Although only small case series have been published to date, they consistently show a correlation between azole resistance and failure to azole therapy. This was most pronounced in a series of Dutch patients with azole-resistant invasive aspergillosis, where the 12 week mortality rate was 88%, which is approximately twice as high as might be expected in patients with azole-susceptible disease.[13] Another problem is that cultures are required to determine the susceptibility phenotype, while a positive culture is obtained in only 30% of patients owing to lack of sensitivity of culture. In most patients, therefore, it remains unknown whether the infection is caused by a resistant isolate.

Direct detection of resistance mechanisms in clinical samples has been used successfully in culture-negative patients.[17,18] One study reported the presence of resistance mechanisms in clinical respiratory samples of 55% of patients with chronic lung diseases, most of which were culture negative,[18] which suggests that resistance might be missed in culture-negative patients. Therefore, molecular tools need to be improved and validated in order to facilitate early detection of resistance mechanisms in routine practice.

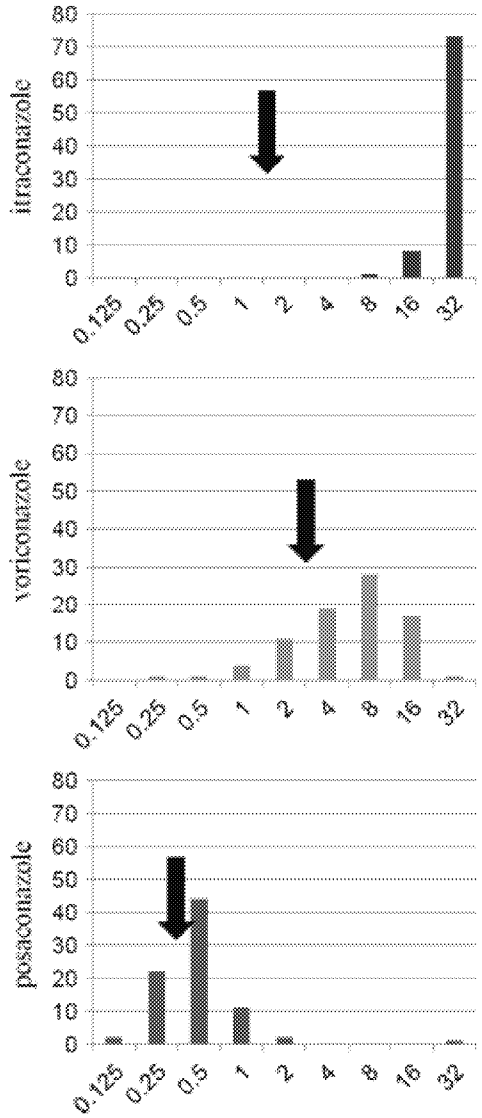

Figure 1. Distribution of minimum inhibitory concentrations (MICs) of a collection of 82 *A. fumigatus* isolates cultured during the Dutch national surveillance study.[13] Agar supplemented with itraconazole was used to identify these azole-resistant isolates. *In vitro* susceptibility testing was performed using a microbroth dilution reference method. The black arrow represents the concentration breakpoint for resistance. All bars to the right of the arrow are isolates that are classified as resistant.

The optimal therapy for azole-resistant *Aspergillus* disease is currently unknown. It appears that the use of azole compounds should be avoided, and alternative regimens that include combination therapy or polyene-based therapy might be effective. More studies are required to determine which approach will be effective.

3 ROUTES OF AZOLE RESISTANCE SELECTION

3.1 The patient route

There are currently two presumed routes of resistance selection. Azole resistance may be selected for in patients during

azole therapy,[19–21] especially in patients that present with aspergilloma or other cavitary lesions. In addition to individual case reports, the largest case series was reported from Manchester in the United Kingdom.[21] All patients had a history of azole therapy, and most were treated for chronic *Aspergillus* diseases, chiefly aspergilloma.[21] This mode of resistance selection was characterised by a high diversity of resistance mechanisms. In cases where multiple isolates were tested over time or multiple colonies from a single sample, various resistance mechanisms were found.[20,21] In some studies, genetic typing of these isolates was performed. Commonly, the first azole-susceptible and the consecutive azole-resistant isolates were genetically the same, indicating that the fungus was capable of acquisition of multiple resistance mechanisms.[20] Furthermore, patients with *Aspergillus* disease due to an azole-resistant isolate showed a high probability of failure to azole therapy.[21] Azole resistance is commonly associated with mutations in the *Cyp51A* gene, which is the target for antifungal azoles. Numerous resistance mechanisms have been described in patients with azole-resistant aspergillosis, which consist of a point mutation or single nucleotide polymorphism (SNP) in the *Cyp51A* gene.[21] Although *A. fumigatus* harbours a *Cyp51A* and a *Cyp51B* gene, thus far only SNPs located in the *Cyp51A* gene have been found to correspond to an azole-resistant phenotype.

Studies in which *A. fumigatus* is exposed to azole compounds under laboratory conditions show a rapid selection of isolates with an azole-resistant phenotype.[22] Genetic analysis showed that some isolates are azole resistant but have no SNPs in the *Cyp51A* gene, indicating a non-*Cyp51A*-gene-mediated resistance mechanism. However, in some azole-resistant isolates, SNPs were found to be identical to those reported in patients treated with azoles.[22]

3.2 The environmental route

In contrast, in the Netherlands, different characteristics were observed: azole-resistant isolates were recovered from azole-naive patients, and a single resistance mechanism was dominant. A recent survey showed that in as many as 64% of patients from whom an azole-resistant isolate was recovered there was no previous history of azole treatment.[13] As *Aspergillus* diseases are not contagious, and patient-to-patient spread is highly unlikely to occur, this observation suggested that the spores that were inhaled by these patients were already azole resistant. This suggests that both azole-susceptible and azole-resistant *A. fumigatus* spores are present in the environment. Furthermore, over 90% of isolates that were recovered from patients harboured the same resistance mechanism, which consisted of a SNP (L98H) in the *Cyp51A* gene in combination with a 34 bp tandem repeat in the gene promoter region (abbreviated as TR_{34}/L98H).[11,13] Recombinant experiments showed that both changes were required for the azole-resistant phenotype.[23] Both above-mentioned observations were highly dissimilar to the Manchester experience and indicated a different route of selection for azole resistance.

It is possible that azole resistance will develop in the ecological niche of *A. fumigatus*, which is soil and compost. This possibility was supported by the recovery of *A. fumigatus* isolates that were resistant to medical triazoles from the environment. Azole-resistant *A. fumigatus* were cultured from soil samples taken from flower beds, compost, leaves and seeds bought at a garden centre.[24] The majority of the environmental azole-resistant isolates harboured the TR_{34}/L98H resistance mechanism.[24] Genotyping of these isolates showed that azole-resistant TR_{34}/L98H isolates originating from the environment and from clinical samples were genetically less diverse compared with control isolates that were azole susceptible.[24]

3.3 A fungicide-driven route?

The question arises as to whether selection of azole-resistant *A. fumigatus* isolates can take place in the environment, i.e. through non-medical application of azole compounds.[25] 14-α-Demethylase inhibitors (DMIs) are commonly used for crop protection, as well as for material preservation. Phytopathogenic moulds are the primary target for fungicides that are used for crop protection, but *A. fumigatus* is not a plant pathogen. Phytopathogenic moulds, however, are exposed to a range of fungicidal chemicals, and through this practice *A. fumigatus* may also be exposed. *A. fumigatus* is a ubiquitous mould that is found in soil, but is found most abundantly in decomposing organic materials such as compost. Airborne spore studies in the proximity of composting sites have shown that up to 75% of the total viable mycoflora captured were *A. fumigatus*.[26] Compared with medical triazoles, the volume of use of DMIs is generally hundreds to a thousand times higher, and the number of different compounds is much greater than available for use for treatment of *Aspergillus* diseases.[27] Azole compounds are also widely used outside agriculture, such as in coatings, sealing kit and materials including leather and wood, in order to prevent fungal growth. It is therefore possible that exposure of *A. fumigatus* and resistance selection could take place through non-agricultural applications of azole fungicides.

Recently, the relation between azole fungicides and selection for azole resistance in *A. fumigatus* was investigated. Thirty-one compounds that had been authorised for use as fungicides, herbicides, herbicide safeners and plant growth regulators in the Netherlands between 1970 and 2005 were investigated for their *in vitro* activity against *A. fumigatus*. Wild-type isolates were investigated, as well as those harbouring the TR_{34}/L98H resistance mechanism.[22] Also, molecule alignment studies were performed in order to identify molecule similarities between fungicides and medical triazoles. The relevance of similarities of the molecule structure was investigated through docking experiments using a homology model of the CYP protein. Although many azole fungicides exhibit no *in vitro* activity against *A. fumigatus*, this study identified five fungicides that showed good *in vitro* activity against wild-type *A. fumigatus* isolates, but not against those harbouring the TR_{34}/L98H resistance mechanism. The five fungicides also exhibited highly similar molecule structure and docking poses compared with the medical triazoles.[22] These fungicides, bromuconazole, tebuconazole, epoxiconazole, difenoconazole and propiconazole, were all from the triazole class and similar to the medical triazoles. Furthermore, they were authorised for use by the Dutch Board for the Authorisation of Plant Protection Products and Biocides between 1990 and 1996, which directly precedes the first recovery of a clinical TR_{34}/L98H isolate in the Netherlands in 1998.[22]

It could be that the use of similar compounds for medical and non-medical applications may result in the selection of resistance mechanisms through one application that affects the use of similar compounds in the other area of use. This has been shown, for instance, for extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli*, where the use of antibacterial agents in food production in animals has selected for this resistance mechanism. The resistant bacteria are transferred to humans through consumption of food containing ESBL *E. coli*, which may then cause infections in humans that are difficult to treat.[28] It

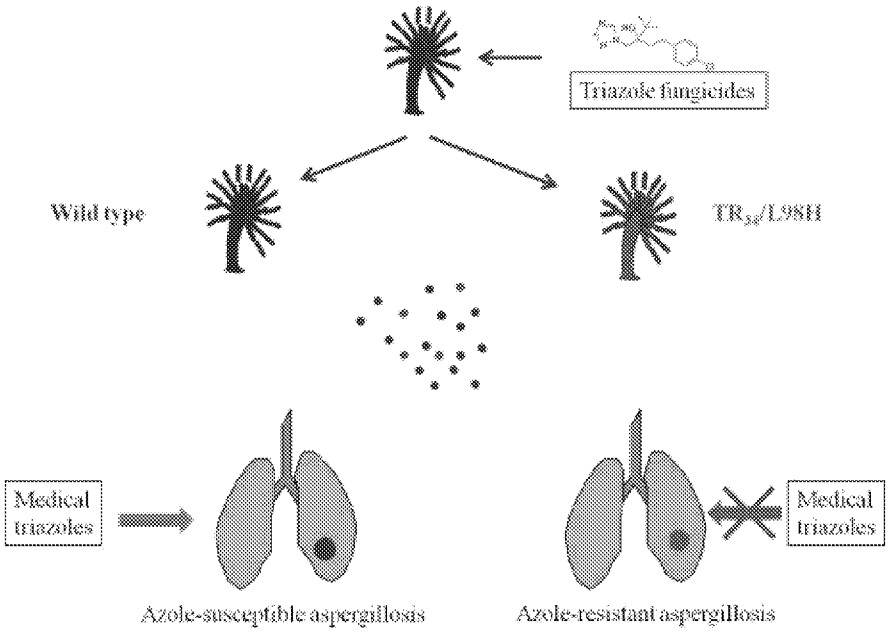

Figure 2. The presumed fungicide-driven route of azole resistance selection in *Aspergillus fumigatus*. Exposure of *A. fumigatus* to certain fungicides may cause selection of resistance mechanisms, such as TR_{34}/L98H, that confer resistance to medical triazoles. Ambient air will contain *Aspergillus* spores with a wild-type phenotype or an azole-resistant phenotype. Patients will inhale both types of conidium, which may cause disease. If the *Aspergillus* disease develops owing to an azole-resistant phenotype, there is a high probability of treatment failure to azole therapy.

appears that a similar phenomenon is now also occurring in fungi and threatens the survival of patients with *Aspergillus* diseases, most notably invasive aspergillosis.

A fungicide-driven route of selection of resistance indicates that wild-type conidia and those harbouring TR_{34}/L98H are present in the ambient air and inhaled by humans (Fig. 2). Although healthy individuals will phagocytose the conidia before any harm can be done, in patients with impaired host defences they may go on to develop an infection. Exposure of these patients to azole drugs may facilitate the selection and growth of the azole-resistant spores, as these then have an advantage over the azole-susceptible conidia. Azole-resistant disease could therefore develop both in azole-naive patients and in those undergoing azole therapy, which has indeed been observed in Dutch patients.[10,11,13]

A fungicide-driven route of selection of resistance has not been proven, and neither is it clear which application of fungicides, i.e. agricultural or non-agricultural, causes resistance selection.

4 IMPLICATIONS

In addition to the lack of apparent risk factors for patients to develop azole-resistant *Aspergillus* diseases, other characteristics of the environmental route of resistance selection are becoming evident. Since the emergence of the TR_{34}/L98H resistance mechanism in 1998, another two resistance mechanisms have been found in *A. fumigatus*, both in clinical and environmental samples. One patient developed osteomyelitis due to a pan-azole-resistant *A. fumigatus* with a 53 bp tandem repeat in the promoter region of the *Cyp51A* gene as the underlying resistance mechanism.[28] An isolate with the same resistance mechanism was later recovered from the environment (unpublished observation). The 53 bp resistance mechanism was recovered from the patient in 2006, and since then has not been found in other azole-resistant isolates. A second resistance mechanism was first recovered from a clinical specimen in December 2009. The resistance mechanism involved a combination of genetic changes, including a 46 bp tandem repeat in the gene promoter region and two substitutions in the *Cyp51A* gene Y156F and T289A (TR_{46}/Y156F/T289A).[29] This resistance mechanism is associated with lack of activity of voriconazole, which is the first-choice treatment option in invasive aspergillosis. As might be expected, since then this resistance mechanism has increasingly been found in clinical azole-resistant *A. fumigatus* isolates from patients in at least six hospitals in the Netherlands.[29] If the environmental route of resistance selection indeed exists, continued azole pressure will result in multiple azole resistance mechanisms developing over time, as shown above. The successful migration of resistance mechanisms may depend on the presence of a fitness cost, as isolates harbouring the resistance mechanism will have to compete with wild-type isolates in the field.

At present, only limited experimental data are available concerning the fitness cost of resistance. It has been shown that azole resistance can come with a fitness cost in a series of four isolates recovered from a patient with chronic granulomatous disease.[30] The first two isolates exhibited an azole-susceptible phenotype, while the latter two were azole resistant. The *in vitro* growth characteristics of the azole-resistant isolates were clearly different from those of the azole-susceptible isolates. The resistant isolates were also less virulent in a murine model of aspergillosis.[30] The azole-resistant phenotype was not due to SNPs in the *Cyp* gene, but the resistance mechanism was recently discovered to be due to a mutation in the *HapE* gene.[31] However, for isolates with *Cyp51A*-mediated resistance, there is no evidence for a fitness cost. Growth characteristics *in vitro* and virulence *in vivo* appear to be similar to those of wild-type isolates.[32] Others have also found that SNPs in the *Cyp51A* gene had no major impact on the haeme environment, thus allowing for resistant mutants to produce ergosterol and retain fitness.[33] In azole-resistant *A. fumigatus*, resistance due to a single SNP in the azole target affected the azole drug affinity, but not the enzyme activity.[34] These studies

are supported by experimental data showing similar virulence of TR_{34}/L98H compared with wild-type isolates,[15,16] and by the persistence of TR_{34}/L98H in the environment. Any fitness cost of TR_{34}/L98H would have resulted in the disappearance of this trait in the field, as competition with wild types would have occurred.

Nevertheless, the emergence of multiple resistance mechanisms over time will increase the prevalence of azole-resistant isolates and complicate the management of patients. In addition to a paucity of treatment options, early diagnosis of azole resistance is also a challenge, especially in a setting of multiple resistance mechanisms. The presence of a tandem repeat appears to be associated with resistance selection in the environment, as resistance selection through patient therapy is characterised by SNPs only.

A second characteristic of the environmental route of resistance selection is the potential for migration of the resistance traits. *A. fumigatus* can reproduce asexually and appears to do this very efficiently. The fungus is ubiquitous and has been recovered from all over the world, including areas with extreme climates. Isolates harbouring TR_{34}/L98H are being increasingly reported from countries other than the Netherlands; it has now been found in multiple European Member States, including the United Kingdom, Spain, Belgium, France, Italy, Austria and Denmark.[35] Recent surveillance studies have also recovered this resistance mechanism in azole-resistant *A. fumigatus* isolates from China and India.[36,37] These observations suggest that azole resistance in *A. fumigatus* has become a global problem.

5 FUTURE RESEARCH

It is important to understand the environmental route of azole resistance selection. Although evidence increasingly points towards the use of certain triazole fungicides as the possible cause, definite evidence is still lacking. Proof for a fungicide-driven route of resistance selection would require laboratory and field studies showing that the TR_{34}/L98H resistance mechanism indeed develops through exposure to triazole DMIs. Under laboratory conditions it was not possible to select for TR_{34}/L98H through repeated exposure of *A. fumigatus* to fungicides. This could be due to the fact that very specific conditions may be required that enable TR_{34}/L98H selection, with respect both to the biology of the fungus as well as to the practice of DMI use in the environment. Alternatively, the selection of TR_{34}/L98H may be a very uncommon event, so that recovering it from laboratory experiments needs a longer timeframe and/or larger sample and population sizes. In addition, the likelihood of the TR mutations to spread through the population may be contingent on other mutations (e.g. specific SNPs in the *Cyp51* gene itself). All these are possibilities and hypotheses that require a systematic and large-scale laboratory programme, crucially supplemented by detailed field studies and surveys.

Between 1998 and 2011, three environmental resistance mechanisms were discovered in the Netherlands, two of which have already spread across the country. Field studies would be required to determine how TR_{34}/L98H develops and the nature of the exposure of *A. fumigatus*, i.e. in the setting of crop protection or in settings not related to agriculture. Understanding the route of azole resistance selection will help to design prevention strategies. Also, the effect of withdrawal of specific fungicides on the prevalence and survival of TR_{34}/L98H within a wild-type population will be important to investigate.

In addition to the above-mentioned studies, international surveillance is warranted. As *in vitro* susceptibility testing is not routinely performed in most clinical microbiology laboratories, the true prevalence of azole resistance is probably underestimated. Furthermore, diagnostic tools that allow timely detection of azole resistance, even in culture-negative cases, are urgently needed. Also, treatment options for the management of azole-resistant disease need to be explored. At present, patients with azole-resistant central nervous system aspergillosis have an especially poor prognosis owing to the fact that the use of the most effective drug, voriconazole, is precluded. Alternative treatment strategies, including combination therapy, should be evaluated *in vitro* and in animal models, in order to have some indication of efficacy in human disease.

Given the prominent role of the azole class in food production safety and in the management of *Aspergillus* diseases, industry and academia should join forces to conduct the necessary research. Only then can evidence-based measures be proposed to relevant bodies, which will benefit the application of these drugs in both areas.

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